10-23-2019

Salt tolerance diversity in diploid and polyploid cotton (Gossypium) species

Yating Dong
Iowa State University

Guanjing Hu
Iowa State University, hugj2006@iastate.edu

Jingwen Yu
Zhejiang University

Sandi Win Thu
Iowa State University

Corrinne Grover
Iowa State University, corrinne@iastate.edu

See next page for additional authors

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Abstract

Development of salt-tolerant genotypes is pivotal for the effective utilization of salinized land and to increase global crop productivity. Several cotton species comprise the most important source of textile fibers globally, and these are increasingly grown on marginal or increasingly saline agroecosystems. The allopolyploid cotton species also provide a model system for polyploid research, of relevance here because polyploidy was suggested to be associated with increased adaptation to stress. To evaluate genetic variation of salt tolerance among cotton species, 17 diverse accessions of allopolyploid (AD-genome) and diploid (A-, D-genome) Gossypium were evaluated for a total of 29 morphological and physiological traits associated with salt tolerance. For most morphological and physiological traits, cotton accessions showed highly variable responses to two weeks of exposure to moderate (50 mM NaCl) and high (100 mM NaCl) hydroponic salinity treatments. Results showed that the most salt tolerant species were the NE Brazilian allopolyploid G. mustelinum, the D-genome diploid G. klotzschianum from the Galapagos Islands, following by the African/Asian, A-genome diploids. Generally, A-genome accessions outperformed D-genome cottons under salinity conditions. Allopolyploid accessions did not show significant differences from either diploid genomic group in salt tolerance, but they were more similar to one of the two progenitor lineages. Our findings demonstrate that allopolyploidy per se need not be associated with increased salinity stress tolerance, and provide information relevant to utilization of the secondary Gossypium gene pool for breeding improved salt tolerance.

Keywords

Allopolyploidy, ecophysiology, evolutionary divergence, salt metabolism, abiotic stress

Disciplines

Agricultural Science | Cell and Developmental Biology | Ecology and Evolutionary Biology | Genetics and Genomics | Plant Breeding and Genetics

Authors

Yating Dong, Guanjing Hu, Jingwen Yu, Sandi Win Thu, Corrinne Grover, Shuijin Zhu, and Jonathan F. Wendel

MS. YATING DONG (Orcid ID : 0000-0002-0268-2995)

DR SHUIJIN ZHU (Orcid ID : 0000-0001-6209-9630)

Article type : Original Article

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Yating Dong[1,2], Guanjing Hu[2], Jingwen Yu[1], Sandi Win Thu[3#], Corrinne E. Grover[2], Shuijin Zhu[1*], Jonathan F. Wendel[2*]

[1]Department of Agronomy, Zhejiang University, Hangzhou, Zhejiang 310058, China

[2]Department of Ecology, Evolution, and Organismal Biology, Iowa State University, Ames, IA 50011, USA

[3]Department of Genetics, Development, and Cell Biology, Iowa State University, Ames, IA 50011, USA

[#]Current address: School of Biological Sciences, Washington State University, Pullman, WA 99164, USA

* Authors for Correspondence:

Jonathan F. Wendel

Tel: +1-515-294-7172 Fax: +1-515-294-1337

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/TPJ.14580

Email: jfw@iastate.edu;

Shuijin Zhu

Tel: +86-13067922851

Email: shjzhu@zju.edu.cn

Running head: Salt tolerance in diploid and polyploid cottons

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Summary

Development of salt-tolerant genotypes is pivotal for the effective utilization of salinized land and to increase global crop productivity. Several cotton species comprise the most important source of textile fibers globally, and these are increasingly grown on marginal or increasingly saline agroecosystems. The allopolyploid cotton species also provide a model system for polyploid research, of relevance here because polyploidy was suggested to be associated with increased adaptation to stress. To evaluate genetic variation of salt tolerance among cotton species, 17 diverse accessions of allopolyploid (AD-genome) and diploid (A-, D-genome) *Gossypium* were evaluated for a total of 29 morphological and physiological traits associated with salt tolerance. For most morphological and physiological traits, cotton accessions showed highly variable responses to two weeks of exposure to moderate (50 mM NaCl) and high (100 mM NaCl) hydroponic salinity treatments. Results showed that the most salt tolerant species were the NE Brazilian allopolyploid *G. mustelinum*, the D-genome diploid *G. klotzschianum* from the Galapagos Islands, following by the African/Asian, A-genome diploids. Generally, A-genome accessions outperformed D-genome cottons under salinity conditions. Allopolyploid accessions did not show significant differences from either diploid genomic group in salt tolerance, but they were more similar to one of the two progenitor lineages. Our findings demonstrate that allopolyploidy *per se* need not be associated with increased salinity stress tolerance, and provide information relevant to utilization of the secondary *Gossypium* gene pool for breeding improved salt tolerance.

Introduction

Soil salinity is a major abiotic stress limiting plant growth and productivity (Munns and Tester, 2008). Although recent statistics for the present global status of soil salinization do not exist (Shahid *et al.*, 2018), saline soils occupied more than 20% of total irrigated area by the mid-1990s (Ghassemi *et al.*, 1995). Since then, the extent of salinity has increased annually due to various reasons such as low precipitation, high surface evaporation, and poor irrigation practices without proper drainage management. Estimates predict that over 50% of all arable land will be salinized

by 2050 (Wang *et al.*, 2003). The excessive amounts of salts in soil, most commonly Na^+ and Cl^-, reduce water potential, disturb ion homeostasis, and cause ion toxicity in plant cells (Sahi *et al.*, 2006; Munns and Tester, 2008; Teakle and Tyerman, 2010). This complex suite of hyperosmotic and hyperionic stresses affect all major aspects of plant physiology and metabolism. Excess NaCl in the soil solution decreases water and nutrient uptake ability, and once Na^+ and Cl^- accumulate to toxic concentrations, both ions adversely affect plant growth and development. Under these circumstances, a wide range of physiological and biochemical responses ensue, including reduction of photosynthesis and respiration rates, inhibition of specific protein expression, production of reactive oxygen species (ROS), and even perturbation of nucleic acid metabolism (Munns and Tester, 2008). Accordingly, developing an understanding of salinity tolerance mechanisms could provide insights for enhancing the salinity tolerance of economically important crops.

Plant species show great variability in their inherent salt-tolerance. For instance, halophytes such as *Atriplex halimus*, *Suaeda maritima*, and *Mesembryanthemum crystalinum* naturally grow in high salt conditions and can complete their life cycles when experiencing up to 200 mM NaCl (Black, 1960; Ushakova *et al.*, 2005; Flowers and Colmer, 2008). Halophytes only constitute around 1% of the world's flora but are phylogenetically widely distributed. In contrast to halophytes, growth of glycophytes such as citrus, tomato, and avocado is salt-sensitive and is largely inhibited by milli-molar Na^+ concentrations (Flowers *et al.*, 2010). For glycophytes, fresh water is essential throughout development. Considerable effort has been directed at investigating salt tolerance in plants, especially for crop species such as wheat (Munns *et al.*, 2006; Yang *et al.*, 2014; Feng *et al.*, 2017), barley (Seckin *et al.*, 2010; Mian *et al.*, 2011; Long *et al.*, 2013), rice (Zeng *et al.*, 2002; Ren *et al.*, 2005; Hu *et al.*, 2006; Reddy *et al.*, 2017; Patishtan *et al.*, 2018; Zhou *et al.*, 2018), and cotton (Gossett *et al.*, 1994; Ashraf, 2002; Meloni *et al.*, 2003; Lv *et al.*, 2008; Pasapula *et al.*, 2011; Yu *et al.*, 2016).

Plants employ several response strategies against salt stress, including accumulation of compatible solutes for osmotic stress, the production of oxygen scavengers against ROS, and the

compartmentalization or excretion of toxic ions to maintain ion homeostasis (Zhu, 2001; Flowers and Flowers, 2005; Flowers and Colmer, 2008). Generally, plant salt tolerance requires the coordinated action of a number of processes, thus complicating our understanding of mechanisms in any particular case. From a crop improvement perspective, traditional breeding strategies have often used a simple visual assessment of salt injury or a proxy (e.g., biomass) to assess salt tolerance. It now is evident, though, that variation in response to salt stress is multifaceted and polygenic, so a more comprehensive approach is necessary (Yeo and Flowers, 1989; Talei *et al.*, 2012; Negrão *et al.*, 2017). To date, several studies have been conducted based on multivariate analyses to screen salt tolerance within different genotypes or accessions of many important plants, such as *Solanum lycopersicum (Manaa et al*., 2011), *Triticum turgidum* (Feng *et al*., 2017), *Andrographis paniculate* (Talei *et al*., 2012), *Oryza sativa* (Pires *et al*., 2015).

Polyploidy (whole genome duplication) in plants has often been associated with ecologically marginal areas or niche expansion (Ehrendorfer, 1980; Stebbins, 1985; Novak *et al.*, 1991; Maherali *et al.*, 2009; Pandit *et al.*, 2011; McIntyre, 2012). A possible example of this is in *Gossypium*, where diploid species are found mostly inland, away from coastal margins, whereas the allopolyploids (truly wild forms) are largely species of littoral environments (Fryxell, 1979). This raises the possibility that allopolyploid cottons possess higher native salt tolerance than do their diploid forbearers. Cotton is an important economic crop that is unrivaled as the natural source of textile fiber. The cotton genus (*Gossypium spp.*) consists of more than 50 species grouped into nine genome groups based on relative chromosome size and behavior during interspecific meiotic pairing, and is widely distributed in the arid to semi-arid subtropical to tropical regions worldwide (Endrizzi *et al.*, 1985; Wendel *et al.*, 2010; Wendel and Grover, 2015; Wang *et al.*, 2018). There are eight monophyletic diploid genome groups (A through G and K) the collectively including at least 46 species, as well as one allotetraploid clade (AD) with seven species (Wendel and Grover, 2015; Gallagher *et al.*, 2017; Wang *et al.*, 2018). Four distinct species have been independently domesticated over the past 6000 years or more, including two Old World Africa-Asian diploids (*G. arboreum* and *G. herbaceum*), which belongs to A-genome, and two New World allopolyploids from the Americas (*G. hirsutum* and *G. barbadense*), which

belong to the AD-genome (Endrizzi *et al.*, 1985; Wendel *et al.*, 2012). The latter now dominate world cotton production, accounting for >95% of global cotton production.

As a moderately salt tolerant crop, the salinity tolerance of cotton has been widely explored (Rodriguez-Uribe *et al.*, 2011; Yao *et al.*, 2011; L. Zhang *et al.*, 2013; Peng *et al.*, 2014; Chen *et al.*, 2016; Zhang *et al.*, 2016; Gong *et al.*, 2017; Wei *et al.*, 2017). Nearly all of these studies are based on 'omics' analyses or physiological attributes of upland cotton (*G. hirsutum*) cultivars, with the rare inclusion of a few wild diploid species. In addition, it has been pointed out there is considerable variation for salt tolerance within and between species (Ashraf, 2002). As the secondary germplasm pool for cultivated cotton, the wild cotton species, especially the putative donors of A- and D- subgenomes, are of special interest, in that they may possess favorable traits for cotton production that tetraploid cultivars lack (Ulloa *et al.*, 2007; Chee *et al.*, 2016). Moreover, it is widely accepted that some polyploid organisms show better tolerance to adverse environmental conditions such as water stress (Maherali *et al.*, 2009), salinity (Yang *et al.*, 2014), and heat (Takahagi *et al.*, 2018) than do their diploid progenitors. For example, studies have shown that allopolyploid *Arabidopsis* (Chao *et al.*, 2013), rice (Tu *et al.*, 2014), citrus (Ruiz *et al.*, 2016) and wheat (Dubcovsky and Dvorak, 2007; Yang *et al.*, 2014) exhibit higher fitness under salinity stress than their diploid counterparts. As an ideal model for plant polyploid research, it is still unclear how much diversity exists in salt tolerance among different *Gossypium* species.

In an effort to generate foundational information on salt tolerance diversity in domesticated cotton and its wild relatives, we studied 17 cotton accessions from three different genome groups, five from the A-genome, six from the D-genome and six from the AD-genome. For each of these accessions, we characterized salt tolerance using 29 key physiological traits, including plant growth characteristics, photosynthesis related variables, ion content variation and antioxidant production. Specifically, we addressed the following questions: (a) How much difference exists in trait performance among different cotton accessions, species, and genome groups under control and salinity conditions? (b) Do the three genome types differ in salt tolerance, and if so, with respect to which traits? (c) Do polyploids differ from their diploid relatives in salinity responses?

Results

To understand the variation in salinity tolerance among *Gossypium* species, twelve diploid and allotetraploid species were assayed, including two A-genome diploids (A_1 - *G. herbaceum* and A_2 - *G. arboreum*), six D-genome diploids (D_1 - *G. thurberi*, D_{3d}- *G. davidsonii*, D_{3k} - *G. klotzschianum*, D_5 - *G. raimondii*, D_6 - *G. gossypioides*, and D_8 - *G. trilobum*), and four AD-genome polyploids (AD_1 - *G. hirsutum*, AD_2 - *G. barbadense*, AD_4 - *G. mustelinum*, and AD_5 - *G. darwinii*) as shown in Table 1 and Figure 1. For the cultivated species A_1, AD_1, and AD_2, two to three accessions were included to survey both the wild forms and representatives of their elite cultivars. Because no truly wild form has ever been found for A_2, two cultivated accessions were used. Two salt treatments representing moderate and high salinity conditions were performed using the hydroponic nutrient solution supplemented with 50 mM and 100 mM NaCl, respectively. These concentrations were selected according to soil categorization by the US Salinity Laboratory (Wallender and Tanji, 2012), corresponding to the soil electrolyte conductivity of 2-8 dS m^{-1} as moderate and >8 dS m^{-1} as high saline conditions. Over the treatment period of two weeks, inhibited growth and smaller, thicker leaves were observed in salt treated plants in comparison with those grown in the control conditions. After 10 days, plants under high salt conditions started to show more severe symptoms, including wilting, burnt-like spots in the center of leaves, and even necrosis; D_5 and D_8 plants gradually died by the end of the treatment period. These observations were consistent in replicated experiments. Thus, the D-genome diploids *G. raimondii* and *G. trilobum* are the least tolerant to high salinity among the surveyed species; accordingly, their salt responses were only studied under the moderate conditions.

Measurement of morphological and physiological changes in response to salt stress

As summarized in Table 2, twenty-nine morphological and physiological traits related to salt tolerance were measured to evaluate salt stress responsiveness. Other traits such as seed germination rate, seeding survival, and root length may also respond to salinity stress (Claeys and Inzé., 2013), but root growth of hydroponic-grown plants is usually little affected by salt tress (Snapp and Shennan., 1992). In this study, these 29 traits are categorized into growth rate and leaf

water status traits, photosynthesis related parameters, accumulation of ions, lipid peroxidation, and antioxidant enzyme activities. For each individual trait, a salt tolerance index (STI) was calculated by the ratio of measurement under stress conditions to the measurement from control plants, except for Na^+ and MDA content, which were calculated in 1/STI. Nearly all accessions showed differences in traits measured at different salinity concentrations and most trait values were down regulated by salt treatments (Figure 2a). Compared with control conditions, most accessions showed higher peroxidase (POD) activity, superoxide dismutase (SOD) activity, chlorophyll a to chlorophyll b ratios (Chla/b), and water use efficiency (WUE), in response to both salt treatments. Likewise, MDA contents in most accessions were lower under salinity conditions. For ion contents, the increments of Na^+ content resulted in decreases of K^+ and Ca^{2+} contents in all tissues but with some exceptions, such as the root potassium content (Root_K), which in $D_{3\text{-}d}$, D_5, A_1-73, A_2-101 and TM1 was higher under 50 mM NaCl than under control conditions. Although the change tendency of most traits was similar between moderate and high salt concentrations, differences still occurred, mainly in some photosynthesis related traits. For instance, five accessions ($D_{3\text{-}k}$, A_2-101, AD_1-Tx665, AD_2-GB0303, and AD_5) showed higher stomatal conductance (Gs) at 50mM NaCl than under control conditions, although all accessions exhibited lower Gs under 100 mM NaCl compared with control conditions. A similar pattern was exhibited for transpiration rate (Tr).

Afterwards, pairwise correlation tests revealed individual STIs were not independent, especially those traits belonging to the same phenotypic category which were often highly correlated with each other (Figure S1). For example, the photosynthesis-related measurements of Gs and Tr were positively correlated at both stress conditions; strong positive and negative correlations were found among the 15 traits measured for ion content in different plant tissues. Thus, principal component analysis (PCA) was performed to calculate a composite salt tolerance index (CSTI) based on all the traits. Under both saline conditions, the first eight principal components (PC1-PC8), which accounted for 88% of the total variance, were used for CSTI calculation, and the corresponding eigenvalues and loading scores of each trait are shown in Tables S1-S3.

Phenotypic divergence and plasticity in response to salt stress between A-, D- and AD-genome cottons

We applied a two-way ANOVA test for each measured trait to compare the salt stress responses of three cotton genome groups (A, D, and AD) grown under different conditions (control, moderate, and high salt treatments). From this analysis three major components of variation were derived: variance due to genome group, variance due to growth conditions, and the variance due to the interaction of genome group with growth conditions. Results from the significance tests are shown in Figure 2b and Table S4. A significant effect of genome group indicates at least one genome group is phenotypically divergent from the others, which was the case for most traits, except those measuring the chlorophyll contents and several photosynthetic parameters, including intercellular CO_2 concentration (Ci), Gs, and WUE. Both growth conditions and interaction effect variances indicate plastic response to salt stress. A significant effect of growth conditions indicates that some of the genome groups have responded to the treatments, and this was universal to all traits investigated except for SOD. A significant interaction effect indicates that there are differences among genome groups, or lineage-specific divergences in stress responsiveness. Traits not exhibiting significant interaction include POD, Chla/b, and most ion contents. In order to specify the patterns of lineage-specific divergence, *post hoc* multiple comparisons were applied to the 18 traits detected with significant interaction effects, with the resulting least square means and significant group separating letters shown in Figure 3.

Under the control conditions, most traits are statistically equivalent between genome groups, except that allopolyploid AD-genome species exhibited lower shoot growth (SG), SOD activity, Root_K, and leaf potassium content (Leaf_K) than did both the diploid A- and D- genome cottons. Under salt treatment conditions, half to two-third of the 18 traits showed significant differences among the three genome groups in response to salinity stress. With respect to plant growth and antioxidant enzyme related parameters, SG and SOD were higher in A- and D-genome cottons than in the AD-genome species across conditions ($P<0.01$). In contrast, leaf relative water contents (RWC) of A-genome cottons under both salt concentrations were significantly lower than those of

D- and AD- genome cottons ($P<0.01$). Compared with D-genome cottons, higher MDA content was observed in A- ($P<0.05$) and AD- ($P<0.01$) genome cottons under 100 mM NaCl treatment, but MDA content in AD-genome cottons did not differ significantly across the three conditions. With respect to photosynthesis parameters and chlorophyll contents, most photosynthesis traits of the three groups of cottons were negatively affected by salt stress. One exception was that WUE of AD-genome cottons increased significantly under 100 mM NaCl ($P<0.01$). Compared with A- and D- genome cottons, the net photosynthetic rate (Pn) of AD-genome cottons was significantly lower under both salinity conditions ($P<0.01$) while Tr showed the more pronounced decrease only under 100 mM NaCl treatment ($P<0.01$). Salt stress did not affect the photosynthesis capacity of A- genome cottons, with the exception of a decrease in Gs and Ci under 100 mM salinity treatment. This suggests that A-genome cottons photosynthesize better under salt conditions than do the other cotton genome groups studied. Total chlorophyll content (Chla+b), chlorophyll a (Chla), and chlorophyll b (Chlb) decreased in the three cotton groups when exposed to both salinity stress treatments, but the Chla/b actually increased in each case. With respect to ion contents, Na^+ exhibited the largest change in all three cotton groups compared to the corresponding controls. Na^+ contents in all three tissues studied (Root_Na, Stem_Na, and Leaf_Na) increased noticeably after exposure to NaCl, and this was accompanied by decreases in K^+ and Ca^{2+} contents, as well as K/Na and Ca/Na ratios. The AD-genome group cottons consistently displayed the lowest concentrations of K^+ and K/Na ratios. Furthermore, the A-genome exhibited relatively higher K/Na ratios. Conversely, no obvious change was found in the Ca/Na ratios of leaf (Leaf_Ca/Na) among the three cotton groups under the two salt conditions.

Salt tolerance ranking of *Gossypium* species and accession

Based on CSTI scores, 17 *Gossypium* species/accessions were ranked for their tolerance to moderate and high salt stresses (Figure 4). A higher CSTI score indicates greater tolerance to salt stress. The most tolerant species at both stress levels is *G. mustelinum*, an allopolyploid species native to a small region of Northeastern Brazil. The least tolerant is a D-genome diploid, *G. trilobum*, which ranked the lowest at the moderate conditions and did not survive the high salt

conditions. At the moderate salt conditions, CSTI scores range from 0.296 to 0.511 among D-genome species and from 0.414 to 0.686 in the AD-genome allopolyploids; a relatively narrower range of scores from 0.516 to 0.659 was found among A-genome diploids. Thus, the range of CSTI scores among allopolyploids spanned from lower to higher than values for the A-genome accessions studied. Similar ranges of variations were observed at the high salt conditions. Between genome groups, A-genome cottons showed significantly higher CSTI scores than D-genome cottons at both conditions (Tukey-adjusted comparisons at 95% confidence level; Figure 5); the CSTI scores of AD-genome cottons appear to be intermediate between those of the two diploid genome groups; however, no significant differences were detected due to the high level of variability within each group (Table S5). Thus, allopolyploid cottons do not necessarily show higher salinity tolerance than the diploid species. For the domesticated species surveyed with both wild accessions and elite cultivars, no significant changes by domestication were detected in their salt tolerance.

Parental contribution of A_2 and D_5 cottons to allopolyploids

Given the multiple trait differences among the three cotton genome groups after salt treatment, the question arises about the progenitor diploid contributions for each trait to that observed in the six allopolyploid cottons investigated. To explore this, the differentiation in the 29 morphological and physiological traits between allopolyploid cottons and their A_2 (*G. arboreum*) and D_5 (*G. raimondii*) parents, the equations $DA_2 = (A_2\text{-AD})/AD \times 100\%$, and $DD_5 = (D_5\text{-AD})/AD \times 100\%$ were used, where DA_2 and DD_5 refer to the trait mean differences between the diploids A_2 and D_5 and the allopolyploid, respectively. Also, the difference between absolute DA_2 and DD_5 values were used to determine whether AD was more similar to A_2 or D_5, or if in fact it was either transgressive (higher or lower) or intermediate. If the absolute value of DA_2 was larger than that of DD_5, AD is inferred to be more similar to D_5 than to A_2, and *vice versa*. In control plants, the majority of the traits of allopolyploid cottons were more similar to A_2 than to D_5 parents, as 78 of 174 trait points (45%) were similar to A_2, 42 trait points (24%) were similar to D_5 and the rest (31%) were intermediate to parental diploids (Figures 6a, b). These relationships flipped after salt

treatments, in that 75 and 42 trait values were more similar to D_5 (43%) than to A_2 (24%) (Figures 6a, c). Under control conditions, RWC was intermediate to progenitor diploid cottons in all allopolyploids. Surprisingly, these values became more A_2-like after treatment with 50 mM NaCl. Interestingly, all of the leaf_K/Na points were converted from A_2-like into D_5-like after salt stress. Under both control and salinity conditions, Root_K and Root_K/Na ratios showed similarity to the D_5 parent, but SOD and Ci were intermediate. These results suggested that allopolyploid cottons display highly variable trait performances relative to their parental diploids in normal and saline environments.

Discussion

Soil salinity is a major factor limiting crop growth and yield, and accordingly, developing salt tolerant cotton cultivars has been a focus in many crop groups, as has been the evaluation of salinity tolerance of diverse germplasm. In the case of cotton, most research has focused on cultivars of the two commercially important tetraploid cottons (*G. hirsutum* and *G. barbadense*). The other two diploid cultivated species, *G. arboreum* and *G. herbaceum*, have been less-studied, but they are still planted in some areas of Pakistan and India with drought prone climates due to their relatively high tolerance to biotic and abiotic stresses (Kulkarni *et al.*, 2009; Sattar *et al.*, 2010; X. Zhang *et al.*, 2013). Consistent with this point, most A-genome cottons investigated here shown relatively high salt tolerance, as judged by trait performance, after being exposed to the moderate and high saline treatments. *Gossypium mustelinum*, an uncommon species, is endemic to a relatively small but seasonally arid region of Northeastern Brazil (Pickersgill *et al.*, 1975). In our study, *G. mustelinum* is the most tolerant species under both salt conditions (Figure 4), notwithstanding the absence of an obvious ecological explanation for this observation (many species studied have similarly arid habitats and/or are more exposed to coastal salt sprays, for example). From a metabolic or physiological perspective, a commonly invoked suggestion is that higher Ca^{2+}/Na^{+} (and/or K^{+}/Na^{+}) ratios and inorganic ions accumulate in roots and shoots of salt-tolerant species, helping them cope with osmotic stress, which can be triggered by both drought and salt treatments. Thus, it seems reasonable to suggest that the dry habitats of *G.*

mustelinum might correspond with the acquisition of salt tolerance because of the physiological correlation and tolerance mechanism overlap between drought and salt stresses. Why this should be more the case for *G. mustelinum* than for other species studied will require further ecophysiological investigation.

From an ecological perspective, evidence is lacking about the adaptive potential of *Gossypium* species to salt stress, although some species are known to occur within areas of active oceanic salt spray (e.g., *G. tomentosum* in Hawaii, *G. darwinii* and *G. davidsonii* in The Galapagos Islands). But in general, there is no obvious ecological or perhaps adaptive explanation for the high level of variability among accessions and species with respect to responses to being challenged with salt (Figure 4). It is an interesting question to consider possible relationships between the composite salt tolerance index and other aspects of the biology and ecology of the species studied here. It is possible that insights might emerge from detailed analyses of the soil conditions and annual water availability in the natural environments of the many species included in the study.

A case in point is the Baja California-Galapagos Island species pair *G. davidsonii* and *G. klotzschianum* (Wendel and Percival, 1990), which performed differently in response to salt stress at the seedling stage (Figure 1a and Figure 4), even though these two taxa have both been mentioned (Wei *et al*., 2017; Zhu *et al.*, 2018) as salt-tolerant species. We note that in previous studies, the maximum treatment time was 144 hours and NaCl concentrations were not increased gradually; it is generally accepted that applying high salt in a single step will cause salt shock (Shavrukov, 2013). It might also be that different adaptive mechanisms may be involved with respect to gradual accumulation versus sudden salt dosing (Carillo *et al.*, 2011). The salt tolerance of some species, even halophytes, may be overridden by a sudden exposure to a high salinity treatment (Albert, 1975). Also, the salt-specific effects during an experiment take time to develop, often spanning several days to a few weeks (Munns, 2002; Munns and Tester, 2008). Plants under salt stress need to maintain ionic homeostasis (in particular Na^+) in the cell cytosol, and this salt-specific phase is essential to discriminate tolerant and sensitive plants (Munns *et al*., 1995; Zhu, 2001). The close relatives *G. barbadense* and *G. darwinii* both displayed good growth

performance under 100 mM NaCl during the first week of treatment, but after about ten days sodium ions started accumulating and burnt-like spots appeared in their leaves. Additionally, although physiological traits might be altered in short-term salt stress studies, this change may not discriminate between salt-tolerant and salt-sensitive individuals, which may not become apparent without longer exposures (Munns, 2002; Higbie *et al.*, 2010).

In our study, *G. klotzschianum* was the most salt tolerant of the D-genome cottons, whereas *G. davidsonii* was near the least (50 mM) or the least (100 mM) tolerant. This is interesting in that these two species are quite similar genetically and share a relatively recent common ancestor (Wendel and Percival, 1990). These observations suggest that salt stress physiological responses might be evolutionarily rather labile, or, phrased alternatively, that salt tolerance might be highly evolvable. *Gossypium davidsonii* tends to be located back from the leading edge of the salt spray zone. Compared with coastal dunes, inland soils commonly have high sulfate salinity (Waisel, 2012), which raises the possibility of a mechanism for salinity stress amelioration. Specifically, the various sulfate forms ($CaSO_4$, Na_2SO_4, and $MgSO_4$) can mitigate the toxic effects of Na^+ and Cl^-, thus enabling plants to survive higher salt conditions (Epstein, 1998; Bressan *et al.*, 1998; White and Broadley, 2003; Shabala *et al.*, 2006; Tuna *et al.*, 2007; Grigore *et al.*, 2012; Waisel, 2012; Köster *et al.*, 2019). For instance, Kent (Kent and Läuchli, 1985) indicated that addition of supplemental Ca^{2+} in NaCl solution helped maintain high K/Na-selectivity in cotton roots, thus offsetting the reduction in root growth caused by NaCl. The identification of a species pair that differ so dramatically in salt stress physiology, as done here for *G. klotzschianum* and *G. davidsonii*, sets the stage for promising comparative investigation of these two taxa and their recombinant derivative lines, with respect to the many interrelated aspects of salt stress physiology and genomics.

It is noteworthy that there was a lack of consistency in the composite salt tolerance index for some accessions between the two salt concentration treatments. We inferred that these accessions respond differently to moderate and high salinity stresses. As in the case of citrus, tetraploids were more tolerant under moderate salt conditions than diploids (Saleh *et al.*, 2008), but diploids

356 outcompeted tetraploids in response to high salinity stress due to the greater accumulation of Na^+
357 and Cl^- in leaves of tetraploids (Mouhaya *et al.*, 2010). Similarly, in a study of *Triticum urartu* and
358 *T. monococcum* grown under moderate salinity, only small variations were found in Na^+ exclusion
359 ability (Gorham *et al.*, 1991), whereas with increased salt stress, variation in the ability of Na^+
360 exclusion was significantly increased in *T. monococcum* (Shah *et al.*, 1987). Excess Na^+ hampers
361 the uptake of K^+ into plant cells and competes with K^+ for binding sites that are important for the
362 catalytic activities of many enzymes (Lazof and Bernstein, 1998; Shabala, 2003; Munns and
363 Tester, 2008). Though there are contrasting reports regarding the uptake and accumulation of Na^+
364 and K^+ in cotton, maintaining higher tissue K/Na and Ca/Na ratios is still thought to be positively
365 correlated with salt tolerance (Cramer *et al.*, 1987; Leidi and Saiz, 1997; Peng *et al.*, 2016; Wang
366 *et al.*, 2017). In our study, the highest K/Na ratio was in leaves of *G. mustelinum,* which might be
367 related to its higher salt tolerance (Figure 1a). Intriguingly, we observed burnt-like injury in leaves
368 of *G. barbadense* and *G. darwinii* after a two-week treatment with 100 mM NaCl, symptoms of
369 ion toxicity (Vijayan *et al.*, 2008; Meng *et al.*, 2011). A high concentration of Na^+ in the
370 cytoplasm can result in degradation of chlorophyll (Ashraf and Harris, 2004). Here, the STIs of
371 Chla+b, Chla, and Chlb of the three accessions were greatly affected by 100 mM NaCl treatment
372 although their values were highest under moderate salt (Figure 1a). Thus, we speculate that severe
373 salt stress might overwhelm the capacity of Na^+ and Cl^- exclusion from the cytoplasm. Varieties
374 that are superior in one attribute associated with salt tolerance may also be inferior or
375 unexceptional in others (Yeo *et al.*, 1990). Therefore, it is not surprising that *G. darwinii* had the
376 highest POD STI, suggesting that this species possesses a stronger antioxidant defense system to
377 mitigate ion toxicity effects compared to *G. barbadense*. Inasmuch as these two species are close
378 relatives, so much so that they were once considered conspecific (Wendel and Percy, 1991), the
379 results presented here provide another example where advanced interspecific recombinant lines
380 may be usefully employed to partition and reveal the various complex physiological and genomic
381 underpinnings of the emergent phenotype of “salt tolerance”.

382 It has long been proposed that polyploid species occupy a wider range of habitats than do their
383 diploid relatives (Lewis, 1979; Ehrendorfer, 1980), perhaps due to higher adaptive flexibility or

plasticity (Otto and Whitton, 2000; Ni *et al.*, 2009). Tetraploid cottons are superior to the diploids in terms of fiber quality and yield, but little is known whether or how the merging of divergent genomes into a common nucleus might enhance adaptation to abiotic stresses. Here we measured the response of four polyploid cotton species to salt stress, i.e., *G. hirsutum*, *G. barbadense*, *G. mustelinum*, and *G. darwinii*. In contrast to the common view of higher tolerance with polyploidy, we found that most tetraploid cottons did not outperform the A-genome diploids under both moderate and high saline conditions (Figures 3, 4). Our findings are consistent with earlier results (Rana, 1986) to a certain extent, where it was shown that diploid Asiatic cottons exhibited higher adaptive responses to salinity than do allopolyploid *G. hirsutum* and *G. barbadense*. Our results are also in line with findings in citrus (Garcia-Sanchez *et al.*, 2002), where it was demonstrated that the diploid sour orange was more salt tolerant than allotetraploid somatic hybrids. These studies, together with ours, indicate that not all allopolyploids have a resistance ability advantage over their diploid progenitors under salt conditions; this, in fact, has been pointed out earlier (Ranney, 2006). It is important to add, however, that artificial hydroponic- or soil-based systems and controlled greenhouses differ from natural environments, and that different developmental stages of plants may behave variably as well.

To the extent that our results for diploid vs. polyploid cotton reflect their inherent physiologies, there are many possible explanations for this observation. One might be that salt stress has not been sufficiently variable among genome types to generate an adaptive response. Another possibility, not mutually exclusive from this adaptationist perspective, is that stress-induced quantitative subfunctionalization is relatively common (Liu and Adams, 2007; Roulin *et al*. 2013; de Carvalho *et al.*, 2014; Ma *et al*. 2015; D'Amelia *et al*. 2018). Based on our comprehensive analysis of 29 morphological and physiological traits, we found that under non-saline conditions, most traits of the AD-genome allotetraploid cottons were more similar to their A_2 (*G. arboreum*) parent than to their D_5 (*G. raimondii*) parent. But under salinity treatments, most traits of tetraploid cottons became more D_5 parent-like. The genomic basis of this phenotypic flip-flop remains unknown, but in principle this is now experimentally addressable given the high quality reference genomes for diploid and tetraploid cottons (Paterson *et al*. 2012; Wang *et al*. 2012; Li *et*

al. 2014; Li *et al*. 2015; Zhang *et al*. 2015; Yuan *et al*. 2015; Liu *et al*. 2015; Du *et al*. 2018; Ma *et al*. 2018; Hu *et al*. 2019; Wang *et al*. 2019). Additional insights into the physiological responses of diploid vs. polyploid cotton may also derive from genic coexpression network analyses, as recently demonstrated for the cotton oilseed (Hu *et al*. 2016) and cotton fiber development (You *et al*. 2016) networks.

In conclusion, the analysis of a suite of phenotypic and physiological traits provides a useful baseline evaluation of salt tolerance among diverse cotton species representing two ploidy levels. We have identified key traits that may contribute to salt tolerance, and species-pairs that differ in salt tolerance and hence are potentially useful experimental materials for future work. In addition, future research linking our findings to GWAS studies into salt tolerance and other abiotic stresses in cotton may prove insightful.

Experimental procedures

Plant materials and salt stress treatment

A total of twelve *Gossypium* species were used in this study, including two A-genome diploids, six D-genome diploids, and four tetraploid AD-genome cotton species. Two to three wild and domesticated accessions were each surveyed for each of the four cultivated species (*G. herbaceum*, *G. arboreum*, *G. hirsutum*, and *G. barbadense*), as detailed in Table 1; their centers of origin or primary geographic distributions are displayed in Figure1. Acid-delinted seeds were germinated on moistened filter paper in petri dishes at 30 °C. After three days, seedlings were transferred into a 1/2 strength Murashige and Skoog (MS) medium solution and grown hydroponically under the same greenhouse conditions at the Pohl Conservatory at Iowa State University. Two-weeks old, uniformly developed seedlings were collected and randomly divided into three treatment groups, one control and two salt stress conditions, with 9-10 seedlings per group. Seedlings of the control group continued to grow in the 1/2 strength MS medium for two weeks. For salt stress treatments, plants were grown in the same nutrient solution supplemented with either 50 mM (moderate) or 100 mM (high) NaCl, for two different salt concentrations per accession. Incremental salt applications were applied to ensure that plants were subjected to salt stress and not to salt shock

(Munns, 2002). Nutrient solutions were closely monitored to avoid solution loss caused by evaporation and frequently replaced during the course of the study.

Morphological and physiological traits analyses

Plant growth and fitness measurements. Plant height (in cm) was measured from the base of hypocotyl to the base of the youngest fully expanded leaf. The shoot growth (SG) of each individual plant was calculated as the difference of plant height before and after salt treatment. To estimate plant water status in response to salinity, the first true leaf of each individual plant was harvested and weighed to determine fresh weight (FW), next immersed in deionized water at 4°C for 24 h to measure turgor weight (TW), and finally oven dried at 70 °C for 48 h to measure dry weight (DW). The relative water content (RWC) was calculated as follows: RWC (%) = (FW-DW)/(TW-DW) x 100% (Schonfeld *et al.*, 1988).

Photosynthesis-related parameters and chlorophyll contents. After two weeks of salt treatment, measurement of photosynthetic parameters was performed on 5-6 seedlings of each accession. Using a portable open flow gas exchange system (Li-6400; LI-COR Biosciences Inc., USA), net photosynthetic rate (Pn), stomatal conductance (Gs), internal CO_2 concentration (Ci), and transpiration rate (Tr) of leaves were determined from 9:00 AM-12:00 PM on the second fully expanded leaves from the apex under photosynthetic active radiation (PAR) of 500 μmol m^{-2} s^{-1}. Each individual plant was measured three times to record technical replicates. Water use efficiency (WUE) was calculated as the ratio of Pn to Tr. To determine chlorophyll content, 100 mg FW of leaf material were cut into small segments and immersed in 5 ml of 80% acetone (v/v) for 48 h in dark. Absorbance of the supernatant was determined spectrophotometrically using a Biotake Synergy™ HT Multi-Detection Microplate Reader (BIO-TEK INSTRUMENTS, INC., USA). The concentrations of chlorophyll a (Chla), chlorophyll b (Chlb), total chlorophylls (Chla+b), and the ratio of chlorophyll a to b (Chla/b) were calculated according to Porra et al (Porra *et al.*, 1989).

Tissue ion contents. Concentrations of Na^+, K^+, and Ca^{2+} were determined for root, stem and leaf tissues after two weeks of stress treatment. Fresh tissues were harvested, rinsed with distilled

water and dilute nitric acid, and subsequently oven dried at 70℃ for 72 h. For each replicate, 100 mg of dry sample was ground into fine powder and digested with concentrated nitric acid and 30% H_2O_2. Ion contents were determined using an Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES, Optima 2100 DV, Perkin Elmer, USA).

Antioxidant enzymes and malondialdehyde. Enzymatic activities were assayed for superoxide dismutase (SOD) and peroxidase (POD). For extraction of enzymes and lipid peroxidation, a total of 500 mg FW of leaf tissue was pooled from seedlings in each replicate, quickly frozen with liquid nitrogen and ground using a mortar and pestle. The ground tissue was homogenized in 5 ml 0.2M PBS buffer (phosphate buffered saline, pH7.0), followed by centrifugation at 12,000 rpm (4℃) for 30 min. The supernatant was collected to determine antioxidant enzyme activities and measure the level of lipid peroxidation. The activity of superoxide dismutase (SOD) was estimated following the procedure developed by Giannopolitis *et al*. (Giannopolitis and Ries, 1977). One unit of SOD activity was defined as the amount of enzyme required cause 50 % inhibition of the rate of formation of nitroblue tetrazolium. The activity of peroxidase (POD) was measured using the guaiacol reduction method (Zhou and Leul, 1999). Malondialdehyde (MDA) content, which is an indicator of membrane lipid peroxidation, was measured with a trichloroacetic acid method described by Zhang et al (Zhang *et al.*, 2014).

Statistical analysis

To compare the phenotypic changes in response to salt stress among A-, D-, and AD-genome cottons, a multiple linear regression model was applied to each trait using the package *lme4* (Pinheiro and Bates, 2000) in the R environment 3.4.3 (Team, 2017). Growth conditions (control, moderate, and high salt treatments with 50 mM NaCl and 100 mM NaCl, respectively), genome group (A, D, and AD), and the interaction of growth conditions with genome group were treated as fixed effects. To ensure the homogeneity of variances, some trait measurements were power-transformed to meet the assumption of normality for regression using the Box-Cox method in the R package *MASS* (Ripley *et al*., 2013). Analysis of variance (ANOVA) was conducted using the R package *car* (Fox and Weisberg, 2011). *Post hoc* multiple comparisons across groups in the

linear model were conducted by Tukey adjusted comparisons at 95% confidence intervals with the package *lsmean* (Lenth, 2015) and *multcompView* (Graves *et al*., 2012), and the estimated least-square means were plotted with 95% confidence intervals using package *ggplot2* (Wickham *et al*., 2008). The pairwise spearman correlation between traits ratios were also determined using the *Hmisc* package (Harrell *et al.*, 2017) and plotted with the *corrplot* package (Wei and Simko, 2017). All heatmaps were generated using the *ComplexHeatmap* package (Gu *et al.*, 2016).

To evaluate the salt tolerance of A-genome, D-genome and AD-genome cottons, principal component analysis (PCA) was utilized to obtain a composite salt tolerance index (CSTI) of each accession under 50 mM and 100 mM NaCl treatments, respectively (Hu *et al.*, 2015; Huang *et al.*, 2016). First, the salt tolerance index (STI) of each trait was calculated as the ratio of the variable measured under salt treatments to the variable measured under control conditions. For MDA content and Na^+ content, which were negatively related with salt tolerance, the ratios were used in traits values under control conditions divided by values under salt treatments. Then, the individual trait STIs were analyzed by PCA with package *FactoMineR* (Lê *et al.*, 2008). Afterwards, the standardized ratios (Z-score) were multiplied by the loading scores in each principal component and comprehensive PC scores were obtained (Tables S1, S2, and S3). PC scores were converted to a 0 to 1 scale with the subordinate function. Finally, the composite salt tolerance indices were generated using the following equations:

(1) The weight: $W_j = \frac{P_j}{\sum_{j=1}^{n} P_j}$, j = 1, 2…, n. W_j indicates that the weight of contribution of component j to total variance percentage.

(2) Degree of subordinate: $u(CI_j) = \frac{CI_j - CI_{min}}{CI_{max} - CI_{min}}$, j = 1, 2…, n. CI_j indicates the no. j of comprehensive index for an accession, CI_{min} and CI_{max} indicate the minimum and maximum values among the no. j of comprehensive indices, respectively.

(3) Comprehensive evaluation value for accession i: $CSTI_i = \sum_{j=1}^{n} [u(CI_j) \times W_j]$, j = 1, 2…, n

To evaluate whether A-genome, D-genome and AD-genome cottons differed in salt tolerance across salt conditions, we used the CSTI (log x+1 transformed to improve normality) of each

accession under 50 mM and 100 mM NaCl as response variables separately in the linear models. The fixed effects included treatments (50 mM NaCl and 100 mM NaCl), genome (A, D and AD), and the interaction treatment:genome.

Acknowledgments

We thank Dr. James W. Raich from Iowa State University for providing the Li-6400 instrument and guidance for photosynthesis measurement. We also thank Kenny McCabe, manager of the Pohl Conservatory at Iowa State University, for assistance with our greenhouse work. Computational support at Iowa State University was provided through the ResearchIT Unit (https://researchit.las.iastate.edu/). The work was funded in part by grants from the NSF Plant Genome Program, Cotton Incorporated, the National Key Technology R&D program of China (2016YFD0101404), and Jiangsu Collaborative Innovation Center for Modern Crop Production. The China Scholarship Council (CSC) provided financial support for Y. Dong to visit Iowa State University.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

Author Contributions

JFW, SZ, and YD designed the research, and GH contributed to refinement of the design. YD, GH, JY, and SWT carried out the experiments. YD, GH, CEG performed the data analyses. YD wrote the manuscript, JFW, GH and SZ contributed substantially to revisions.

Data Availability Statement

Data supporting the findings of this work are accessible within the paper and its Supporting Information files. All other data generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Supporting Information

Figure S1. Correlogram of Spearman correlation among 29 morphological and physiological traits in cottons under 50 mM NaCl (a) and 100 mM NaCl (b) treatments.

Table S1 Eigenvalues of principal components extracted and their contribution and accumulative contribution

Table S2 Loading scores of each trait in principal components extracted

Table S3 Principal component score and composite salt tolerance indices (CSTI) of cotton accessions under salt stress

Table S4 ANOVA analysis of effects of Genome (A vs. D vs. AD) and treatment (Control vs. 50mM NaCl vs. 100mM NaCl) on 29 measured traits in *Gossypium*

Table S5 Difference in salt tolerance among cottons from the A, D and AD genome groups

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935 **Table 1** List of *Gossypium* species and accessions screened for salt stress response.

Accession	Species	Genome	Geographic origin
G. thurberi	*G. thurberi* Todaro	D_1	Mexico and SW U.S.
G. davidsonii	*G. davidsonii* Kellogg	$D_{3\text{-}d}$	Mexico
G. klotzschianum	*G. klotzschianum* Andersson	$D_{3\text{-}k}$	Galapagos Islands
G. raimondii	*G. raimondii* Ulbrich	D_5	Peru
G. gossypioides	*G. gossypioides* (Ulbrich) Standley	D_6	Mexico
G. trilobum	*G. trilobum* (DC.) Skovsted	D_8	Mexico
A_1-52†	*G. herbaceum* Linnaeus	A_1	Africa-Asia
A_1-73			
A_1-Wagad†			
A_2-101†	*G. arboreum* Linnaeus	A_2	Asia
A_2-1096†			
TM1†	*G. hirsutum* Linnaeus	AD_1	Southern Mexico
Tx665			
PS7†	*G. barbadense* Linnaeus	AD_2	NW South America
GB0303			
G. mustelinum	*G. mustelinum* Miers ex Watt	AD_4	Brazil
G. darwinii	*G. darwinii* Watt	AD_5	Galapagos Islands

936 † Cultivated accessions.

Table 2 The 29 morphological and physiological traits related to salt stress measured in this study.

Category	Trait	Description (unit)	Function	References
Plant growth and leaf relative water content	Shoot growth (SG)	The difference of plant height before and after salt treatment (cm)	SG is a sensitive indicator of stress tolerance.	Claeys *et al*., 2014
	Leaf Relative water content (RWC)	It estimates the current water content of the sampled leaf tissue relative the maximal water content it can hold at full turgidity	RWC reflects the plant water status in terms of the physiological consequence of cellular water deficit.	Negrão *et al*., 2017
Photosynthesis related	Net photosynthetic rate (Pn)	The net photosynthetic rate (μmol CO_2 m^{-2} s^{-1})	With more open stomata allowing greater conductance, and consequently indicating that	Chaves *et al*. 2009; Lakshmi *et al*., 1996;

parameters and chlorophyll contents	Stomatal conductance (Gs)	The stomatal conductance (mol CO_2 m^{-2} s^{-1})	photosynthesis and transpiration rates are potentially higher. Ci reflects CO2 assimilation rate. When exposed to salinity treatment, the Pn, Gs, Ci, Tr, and WUE of plants were reduced, and a set of enzymes have been shown to decrease their activity including Rubisco, sucrose phosphate synthase, and nitrate reductase. Salt tolerance species usually shown higher Pn, Gs, Tr, Ci, and WUE in response to salt stress than sensitive ones.	Meyer & Genty, 1998
	Internal CO_2 concentration (Ci)	The CO_2 concentration in mesophyll cells (μmmol·mmol)		
	Transpiration rate (Tr)	The transpiration rate (mmol H_2O m^{-2} s^{-1})		
	Water use efficiency (WUE)	Photosynthetic water-use efficiency, which is defined as the ratio of the rate of carbon assimilation (Pn) to the Tr (μmol CO_2 $mmol^{-1}$ H_2O)		

	Chlorophyll a (Chla)	Leaf chlorophyll a content (mg g^{-1} FW)	Chlorophyll is fundamental to photosynthesis in plants. Chla is a specific form of chlorophyll used in oxygenic photosynthesis while Chlb helps in photosynthesis by absorbing light energy. Plants possess more Chla than Chlb in any situations. In salt-stressed plants, oxidative stress and the inhibition of chlorophyll synthesis induce the decrease in chlorophyll levels. But the chla/b tends to increase due to greater reduction in Chlb compared to Chla.	Ashraf & Harris, 2013; Smirnoff, 1996; Santos, 2004
	Chlorophyll b (Chlb)	Leaf chlorophyll b content (mg g^{-1} FW)		
	Total chlorophyll content (Chla+b)	Total chlorophyll content in leaf (mg g^{-1} FW)		
	The chlorophyll a-to-chlorophyll b ratio (Chla/b)	The ratio of Chla to Chlb in leaf		
Ion contents	Root K^+ (Root_K)	Root potassium content (mg g^{-1}DW)	In plant cells, low Na^+ and high K^+ in the cytoplasm are essential to maintain a cascade of biochemical processes. Ca^{2+} is an important salt tolerant parameter involved in membrane stability. Reducing Na^+ while maintaining high K^+ and Ca^{2+} level is a key factor in determining	Conn & Gilliham, 2010; Dubcovsky *et al*., 1996; Munns 2002; Shabala S & Cuin TA, 2008; Unno *et al*., 2002
	Root Na^+ (Root_Na)	Root sodium content (mg g^{-1}DW)		
	Root Ca^{2+} (Root_Ca)	Root calcium content (mg		

	$g^{-1}DW$)	the ability to tolerate salinity. The capacity of plants to maintain a high cytosolic K^+/Na^+ and Ca^{2+}/Na^+ ratio is likely the key determinants of plant salt tolerance. The uptake and long-distance Na^+ transport in cells is crucial in plants adaptation to salt stress. In most crop species, NaCl accumulation within photosynthetic cells incurs a larger cost than accumulation in root cortical cells. During salt stress, Na^+ is excluded from shoots and K^+ and Ca^{2+} are accumulated in shoots, thereby stabilizing the high cytosolic K^+/Na^+ and Ca^{2+}/Na^+ ratio especially in leaves.
Root K^+/Na^+ (Root_K/Na)	The ratio of potassium content to sodium content in root	
Root Ca^{2+}/Na^+ (Root_Ca/Na)	The ratio of calcium content to sodium content in root	
Stem K^+ (Root_K)	Stem potassium content (mg $g^{-1}DW$)	
Stem Na^+ (Root_Na)	Stem sodium content (mg $g^{-1}DW$)	
Stem Ca^{2+} (Root_Ca)	Stem calcium content (mg $g^{-1}DW$)	

Stem K^+/Na^+ (Root_K/Na)	The ratio of potassium content to sodium content in stem
Stem Ca^{2+}/Na^+ (Root_Ca/Na)	The ratio of calcium content to sodium content in stem
Leaf K^+ (Root_K)	Leaf potassium content (mg $g^{-1}DW$)
Leaf Na^+ (Root_Na)	Leaf sodium content (mg $g^{-1}DW$)
Leaf Ca^{2+} (Root_Ca)	Leaf calcium content (mg $g^{-1}DW$)
Leaf K^+/Na^+ (Root_K/Na)	The ratio of potassium content to sodium content in leaf

	Leaf Ca^{2+}/Na^{+} (Root_Ca/Na)	The ratio of calcium content to sodium content in leaf		
Antioxidative enzymes and lipid peroxidation	Superoxide dismutase (SOD)	The SOD content (U g^{-1}FW)	When plants are exposed to salt stress, the reduced rate of photosynthesis increases the formation of reactive oxygen species (ROS) which in turn result in cell membrane lipid peroxidation. SOD and POD are key enzymes that can remove and detoxify excess ROS in the cells. MDA is the main product of membrane lipid peroxidation that represents the degree of cell membrane damage.	Ozgur *et al*., 2013
	Peroxidase (POD)	The POD activity (U g^{-1} min^{-1})		
	Malondialdehyde (MDA)	The MDA content (μmol g^{-1}FW)		

Figure legends

Figure 1. Centers of origin or primary geographic distributions of the 12 cotton species screened for salt tolerance.

Figure 2. Salt tolerance index (STI) of 29 morphological and physiological traits in 17 cottons (a) and two-way ANOVA analysis of traits values between A-, D-, and AD- genome cottons (b). Numbers in each cell represent STI values; * represents $P<0.05$, ** represents $P<0.01$, and ns means non-significant (two-way ANOVA).

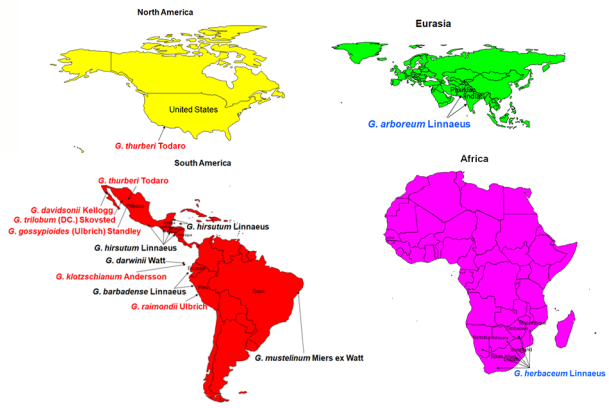
Figure 3. A-genome, D-genome and AD-genome *Gossypium* differ in 18 morphological and physiological traits. The least-squares mean ± 1 SE of each trait are plotted for each genome group under each conditions (x-axis, arranged from control conditions to moderate and high NaCl concentrations), estimated from linear models where the response variables were power-transformed if necessary (sqrt, square root; cube, cube root; log, natural logarithm). Different capital letters indicate significant differences among genome groups for the same salinity treatment; different lowercase letters indicate significant differences among salinities for the same genome group (Tukey-adjusted comparisons at 95% confidence intervals).

Figure 4. Comprehensive salt tolerance index (CSTI) of each species/accession in response to 50 mM and 100 mM NaCl respectively.

Figure 5. AD-genome cottons do not show higher salt tolerance compared with A- and D-genome cottons. The composite salt tolerance index, which was calculated by PCA analysis, was transformed (log x+1) in the linear model. The least-squares means ± 1 SE are plotted for each genome group under each conditions. Different letters mean significant difference at 95% confidence intervals by Tukey-adjust multiple comparisons.

Figure 6. Differentiation in morphological and physiological traits between allotetraploid cottons and their A_2 and D_5 parents. All measured traits in the six tetraploid cottons under control and 50 mM NaCl treatment (a). Relative proportions of the three situations, i.e., intermediate to both

progenitor parents, similar to A_2 parent and similar to D_5 parent under control conditions (b) and 50 mM NaCl treatment (c). SG, shoot growth; RWC, leaf relative water content; Pn, net photosynthetic rate; Gs, stomatal conductance; Ci, intercellular CO_2 concentration; Tr, Transpiration rate; WUE, water use efficiency; Chla, chlorophyll a content; Chlb, chlorophyll b content; Chla+b, the total of chlorophyll a and chlorophyll b; Chla/b, the chlorophyll a to chlorophyll b ratio; Root_K, Root K^+ content; Root_Na, Root Na^+ content; Root_Ca, Root Ca^{2+} content; Root_K/Na, the K to Na ratio in root; Root_Ca/Na, the Ca to Na ratio in root; Stem_K, Stem K^+ content; Stem_Na, Stem Na^+ content; Stem_Ca, stem Ca^{2+} content; Stem_K/Na, the K to Na ratio in stem; Stem_Ca/Na, the Ca to Na ratio in stem; Leaf_K, Leaf K^+ content; Leaf_Na, Leaf Na^+ content; Leaf_Ca, Leaf Ca^{2+} content; Leaf_K/Na, the K to Na ratio in leaf; Leaf_Ca/Na, the Ca to Na ratio in leaf; POD, peroxidase; SOD, superoxide dismutase activity; MDA, malondialdehyde content.



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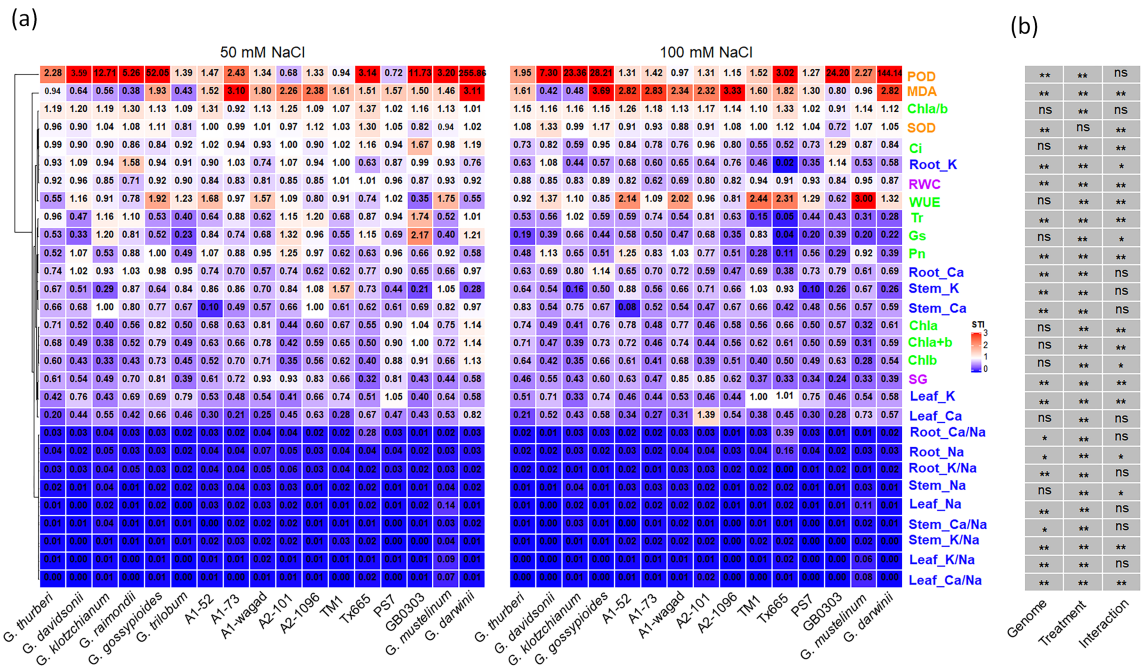

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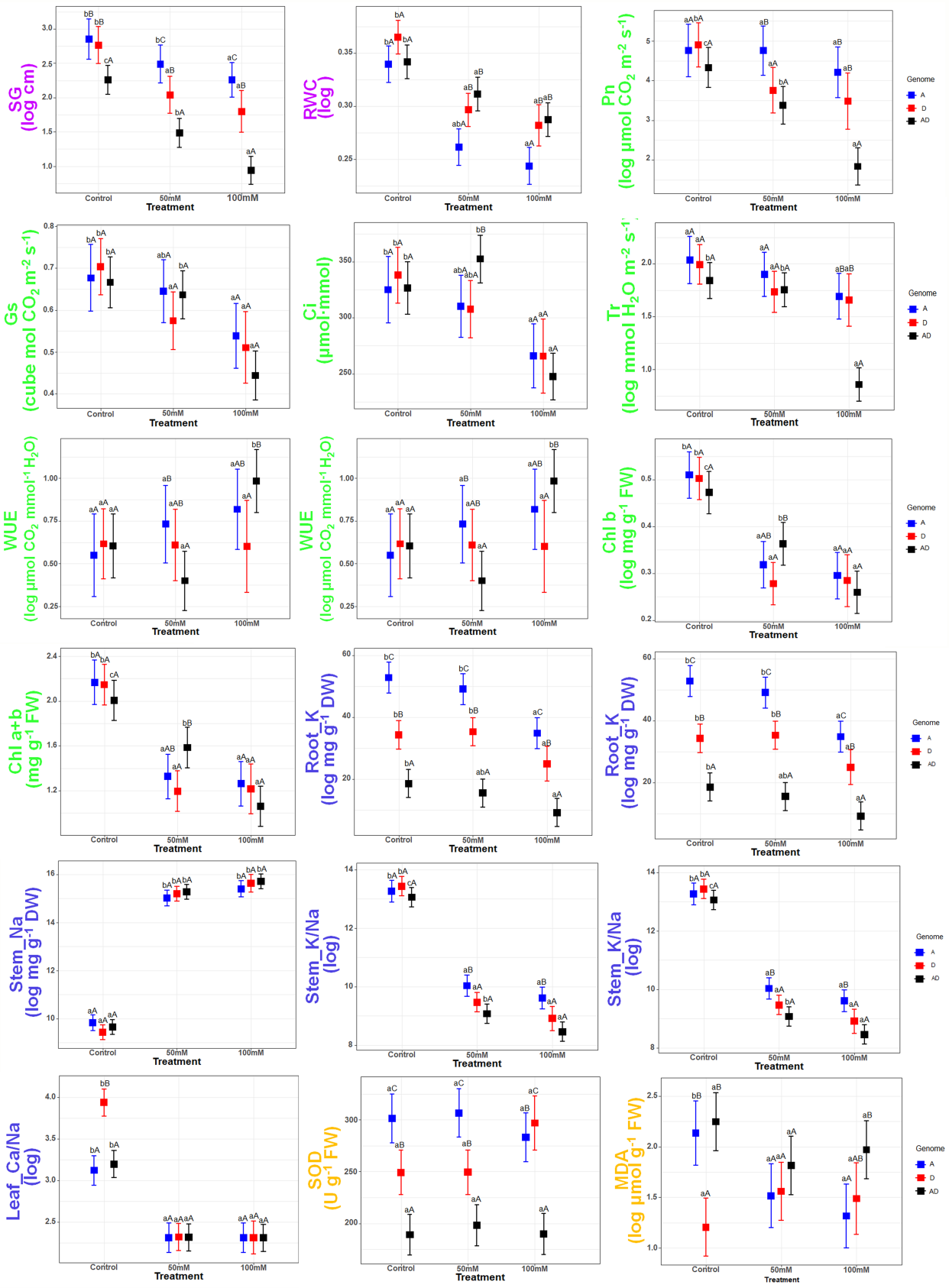

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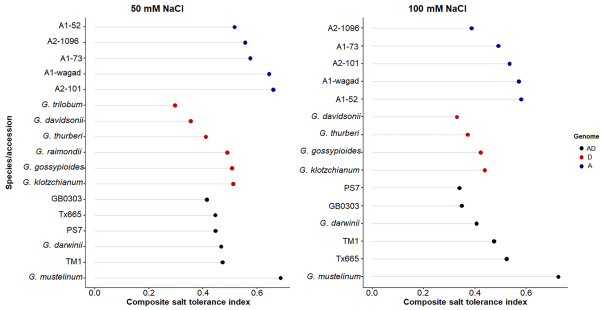

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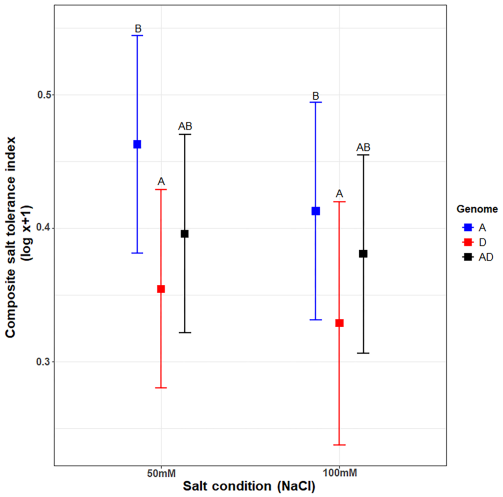

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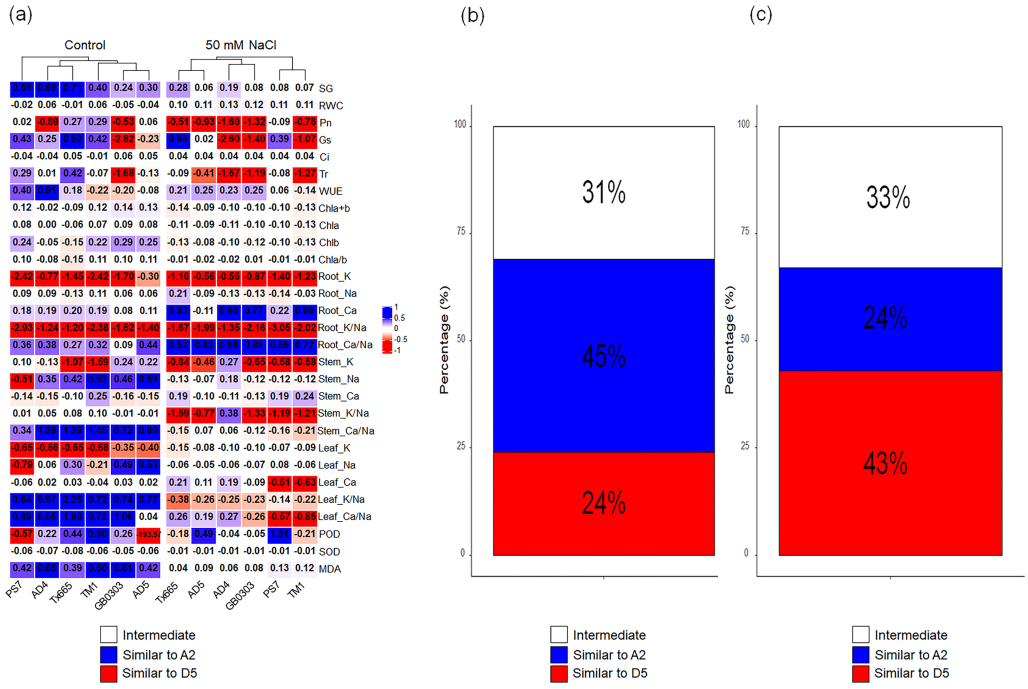

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